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14. ABSTRACT
An optical imaging system was developed to allow the visualization of novel NIR-tagged radiological contrast agents. A fluorophore-tagged ligand based on alendronate sodium was developed and tested in mice. The system showed strong affinity for physiological calcium and could be further developed as a radiological contrast agent for x-ray mammography.

15. SUBJECT TERMS
Breast cancer; radiological imaging; contrast agents; bisphosphonate; mammography

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1. Introduction

In radiological imaging of the breast, the appearance, patterns and structures of mammary microcalcifications—deposits of calcium oxalate and calcium hydroxyapatite (HA)—are the most significant indicators of benign and malignant breast lesions[1]. X-ray mammography is currently the principal imaging modality applied in the detection and diagnosis of breast diseases. Because of its unique ability to reveal microcalcifications, mammography can detect small carcinomas at an early stage. Although rarely employed in breast imaging, contrast agents are routinely administered for clinical MR and x-ray imaging. Administered intravenously, either prior to or during the procedure, contrast agents enhance the signal in a physiologically-dependent manner and thus extend the diagnostic utility of the procedure. Alendronate, an amino bisphosphonate, is an ideal candidate to be employed as a calcium targeting ligand. Bisphosphonates are stable, minimally toxic, synthetic analogs of pyrophosphate that bind to HA[2]. This work involves the development of biochemical markers for calcium that can serve as contrast agents for advanced radiological procedures and the development of apparatus for in vivo evaluation of these experimental compounds in small animals.

Since 2000, our laboratory has been involved in the development of novel imaging mechanisms and strategies for application in small animals. A particular focus has been on the development of ligands with optical tags and visualization with near infrared optical imaging techniques. The work described here was undertaken in conjunction with a Department of Defense Concept award granted under the Breast Cancer Research Program, for the period of September 2003 through February 2006. Funding under this program has enabled us to develop and evaluate certain calcium targeting strategies in small animal imaging applications. We are very grateful for the BCRP support which covered the critical concept development phase of early-stage research and has enabled us to launch several projects that have been submitted for NIH support.

1.1 Achievement of Objectives

The original broad objective, as stated in the application, was "**to develop biochemical markers for calcium that serve as contrast agents in advanced imaging procedures."** To this end, we have been true to our objective and have achieved a number of important accomplishments – and more. In the original formulation of the project however, we envisioned the immediate development of two novel contrast agents followed by rapid *in vivo* assessment. The proposed statement of work listed two main tasks: 1) the synthesis of gadolinium-based and molybdenum-based bisphosphonates; and 2) evaluation of these agents in experimental animals. As laid out, the path was enormously ambitious and probably somewhat naïve. In addition, the statement of work was problematic in its serial design – task 2 depended on the success of task 1 – and contained no fall-back in the event of failure to achieve certain milestones. Task 1, the synthesis of completely new compounds proved to be much more complex than we originally anticipated and ultimately could not be accomplished with the personnel and resources available.

Given these eventualities, strictly applying the yardstick of the original statement of work, the detailed objectives might appear to fall short of the original goals. However this would be an unfortunate, very limited and flawed means to assess the project accomplishments. As we pursued the overall objectives of this project, as proposed, important and productive accomplishments were attained. These accomplishments, while not laid out in advance in the statement of work, are not only valuable in and of themselves, but do nonetheless bring closer the realization of those detailed tasks.

In this report we describe the major accomplishments of this project which fall into three areas: 1) imaging system developments; 2) optimization of an alendronate-based dye for imaging biological cal-

cium *in vivo*; and 3) development of novel fluorescent imaging techniques for application in cancer research.

2. Body

Recently there has been a great deal of research activity directed towards the development of biomedical imaging techniques that employ near infrared (NIR) light. The absorption coefficient for photons propagating in biological tissue depends strongly on wavelength. This is illustrated in Figure 1. Photons in the wavelength range spanning the NIR window, approximately 700 nm to 900 nm, are highly penetrating in tissue. Light of shorter wavelengths, particularly for the blue-green portion of the visible spectrum (400–550 nm), is absorbed strongly by hemoglobin and light of longer wavelengths is absorbed by water. The NIR window is therefore ideally suited to exploitation in small animal optical imaging.

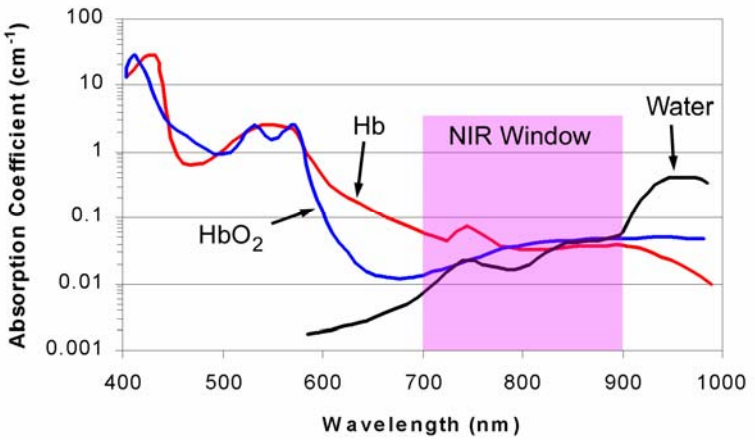

Figure 1: Biological tissue is relatively transparent in the near infrared (NIR) region of the electromagnetic spectrum, i.e. wavelengths between approximately 700 nm and 900 nm. Absorption by hemoglobin and deoxyhemoglobin become appreciable at lower wavelengths and absorption by water dominates at higher wavelengths.

2.1 Imaging System Developments

We have developed an *in vivo* imaging system optimized for fluorescent optical imaging. The imaging system, illustrated in the block diagram and photos of Figure 2, employs epi-illumination via a 450 W Xe arc lamp (Oriel) attached to a condenser, heat-removal filter and lens. Wavelength selection is by interference filters (Omega Optical, custom order) mounted in a filter wheel. Filter spectral characteristics are shown in the graph of Figure 2B overlaid with the absorption and emission spectra of 10 μM indocyanine green, a common NIR fluorescent compound. Excitation light at the output of the filter is deflected by a mirror and directed at a 10 cm square stage. Excitation illumination intensity is approximately 2 mW/cm^2. Emission filtration is achieved by a longpass optical interference filter with cutoff at 798 nm. Filtered emission light is collected by a fixed focal length lens (Tamron, 25 mm, F/1.6) mounted 320 mm above the stage and digitized by a high-resolution, 1280 × 1024 × 12-bit pixel, cooled, 2/3" format CCD, digital camera (ORCA-ER, Hamamatsu). Motor control and image acquisition hardware is interfaced to a Pentium III-based personal computer running system integration and automation software (National Instruments LabVIEW 6). Imaging components are mounted on an optical bench (Kinetic Systems 9100 series). Images shown in the subsequent subsections were acquired with the lens aperture set to F/1.6 and in the full resolution (1280 × 1024 pixels), non-binning, frame blanking acquisition mode of the Hamamatsu camera. Images were processed and manipulated using custom

software running on MATLAB Release 12.1 (Mathworks). Further image manipulation, compression to 8-bit scale and annotations are performed with Photoshop 6 (Adobe).

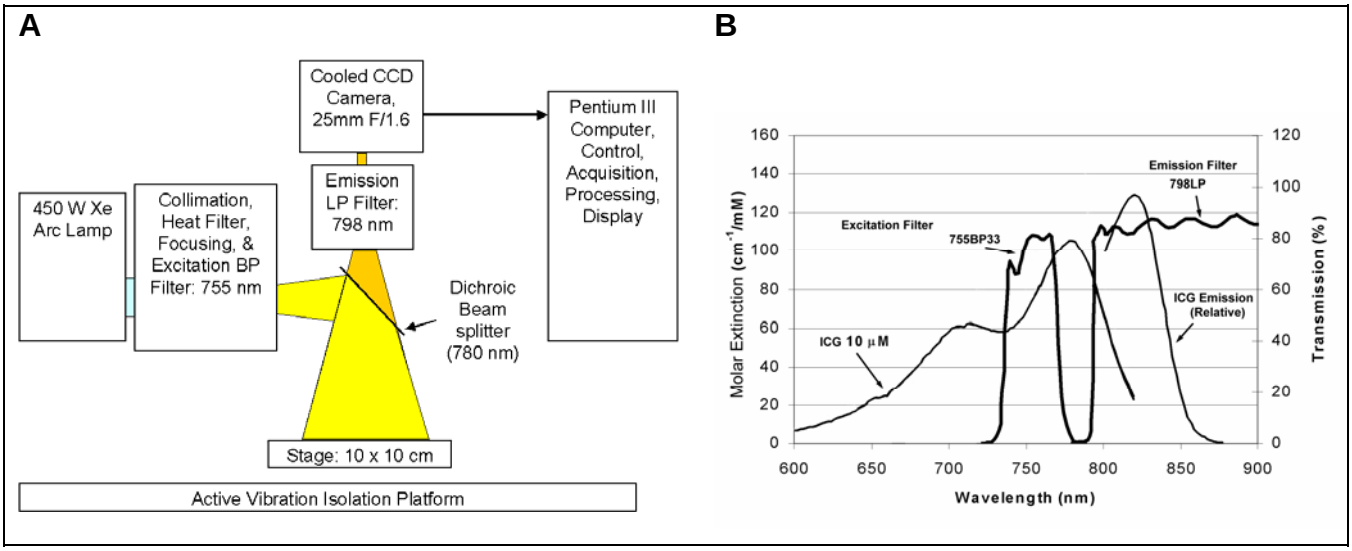

Figure 2: NIR Fluorescent imaging system design. (A) Block diagram showing major system components. (B) Spectral response of NIR bandpass excitation filters and long-pass emission filter, overlaid with the absorption and emission characteristics of indocyanine green.

2.2 Fluorescent Imaging with Alendronate

Bisphosphonates, characterized by two phosphate groups separated by a single carbon atom, are chemical analogs of pyrophosphates (P-O-P linkage) and imidodiphosphates (P-N-P). Biologically, bisphosphonates bind to hydroxyapatite. Technetium labeled aminohydroxypropylidene-bisphosphonate (ADP) and methylene-bisphosphonate (MDP) are commonly used in nuclear medicine as bone scanning agents [3]. Clinically, bisphosphonates inhibit bone resorption and are prescribed for the prevention of osteoporosis [4] and in the palliative treatment of patients with bone metastases [5]. Two such commercially available compounds are pamidronate disodium, available as Aredia® from Novartis, and alendronate sodium, available as Fosamax® from Merck.

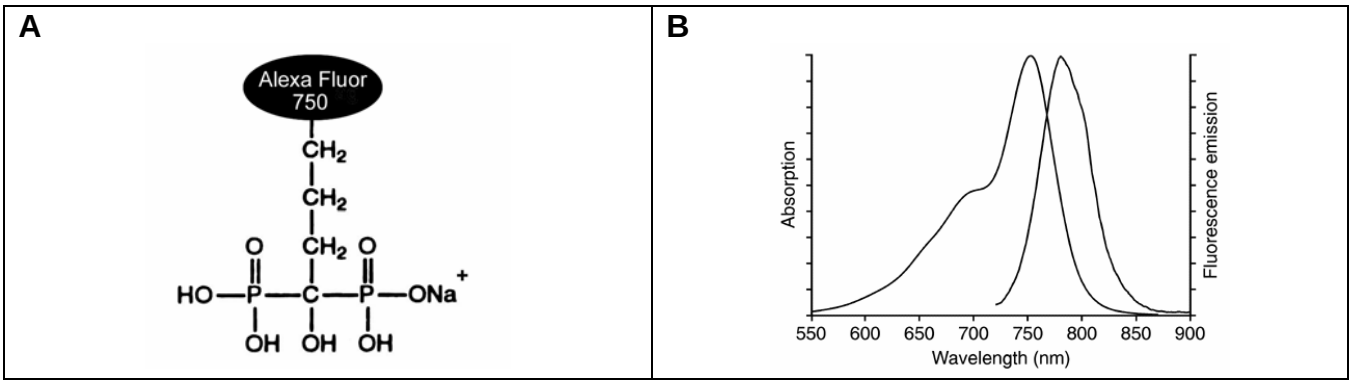

Figure 3: NIR fluorescent bone scanning. (A) Molecular structure of optical bone scanning agent consisting of alendronate sodium conjugated to Alexa Fluor 750 NIR dye. (B) Relative absorption and fluorescence emission spectra for Alexa Fluor 750.

We acquired a small quantity of the bisphosphonate alendronate sodium (Fosamax®) through an agreement with Merck Pharmaceuticles. We prepared a NIR fluorescent analog by reacting alendronate sodium with Alexa Fluor 750 (Molecular Probes Inc.) NIR dye. The structure of this compound is shown in Figure 3A and the absorption and fluorescence emission spectra are shown in Figure 3B. This

alendronate-Alexa Fluor reaction has superior yield (>70%) to the 18-21% yield for pamidronate-IRDye-78 (LI-COR) conjugation reported previously [6].

Representative images are shown in Figure 4 of a mouse approximately 4 hours after intravenous administration of 2.5 nanomoles of the alendronate-Alexa Fluor 750 compound. The animal was imaged while under anesthesia. The bone structure is clearly visible in the images, particularly near superficial skeletal features.

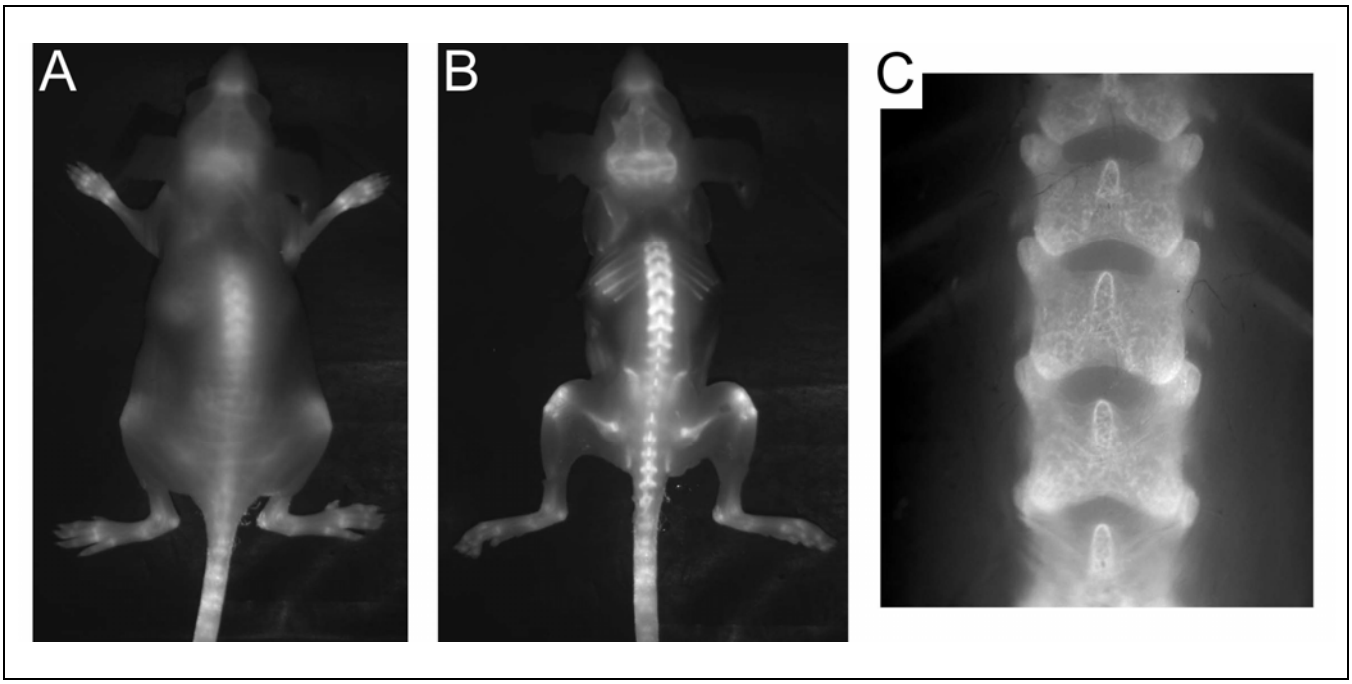

Figure 4: NIRF images of a mouse 4 hours post intravenous administration of 2.5 nanomoles of a developmental bisphosphonate compound whose structure is shown in Figure 5A. (A) Dorsal view, (B) autopsy, skin removed, (C) skin removed, magnified view of vertebral bodies.

2.3 Development of New Techniques

2.3.1 Ex-vivo, Serial Rat Bladder Imaging of Cancer Growth and Response

Our collaborators at Children's Hospital Boston have developed a rat bladder *ex vivo* culture system for tumor cell implantation. Cancer cells are labeled with Qdots-655 using the proprietary Qtracker protocol (Quantum Dot Corporation, Hayward, CA), prior to instillation into the rat bladder. Bladders are harvested 2 days after tumor cell implantation and then maintained in culture for up to twenty days. We refined our imaging system apparatus and techniques to allow for serial imaging as a means to examine tumor growth and response.

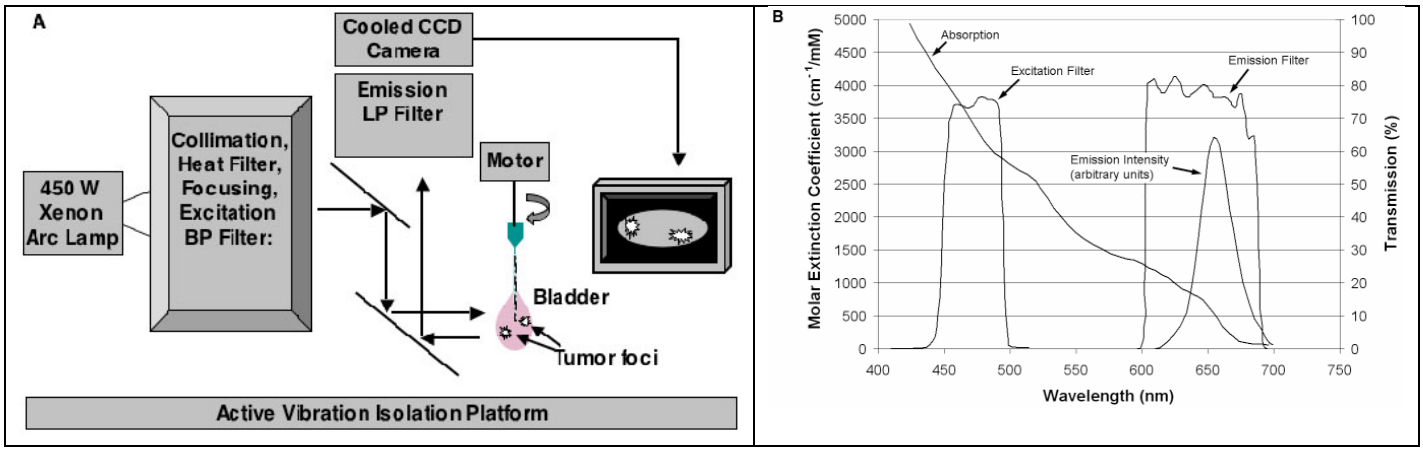

Figure 5: (A) Block diagram showing major system components and set-up for imaging bladders. (B) Transmission curves for excitation and emission filters are shown, overlaid with the absorption and emission characteristics of the Qdot 655 nanocrystals.

Figure 5A shows the block diagram of the system modified for *ex vivo* bladder imaging experiments. The bladder containing the culture is suspended on a spindle which is attached to a stepper motor. The bladder rotation angle is controlled by the computer, which is programmed for an automated, repeatable collection sequence. Excitation wavelength is selected by a bandpass filter with the pass-band set to 450-500 nm to provide efficient excitation of the quantum dot cell-tracking dye. Absorption and emission characteristics of the dye are shown in the graphs of Figure 5B.

Figure 6 shows results in brief from an experiment undertaken with the system. As can be seen in the figure, tumor cell masses were evident in the bladder wall by day seven of organ culture. Several of the lesions had increased in size by day 14, with a ~4-fold increase in tumor burden at 14 days compared to 7 days. Selected tumor regions were observed to decrease in area following 4 days of intravesical instillation of thiotepa, an alkylating agent used for treatment of superficial bladder cancer.

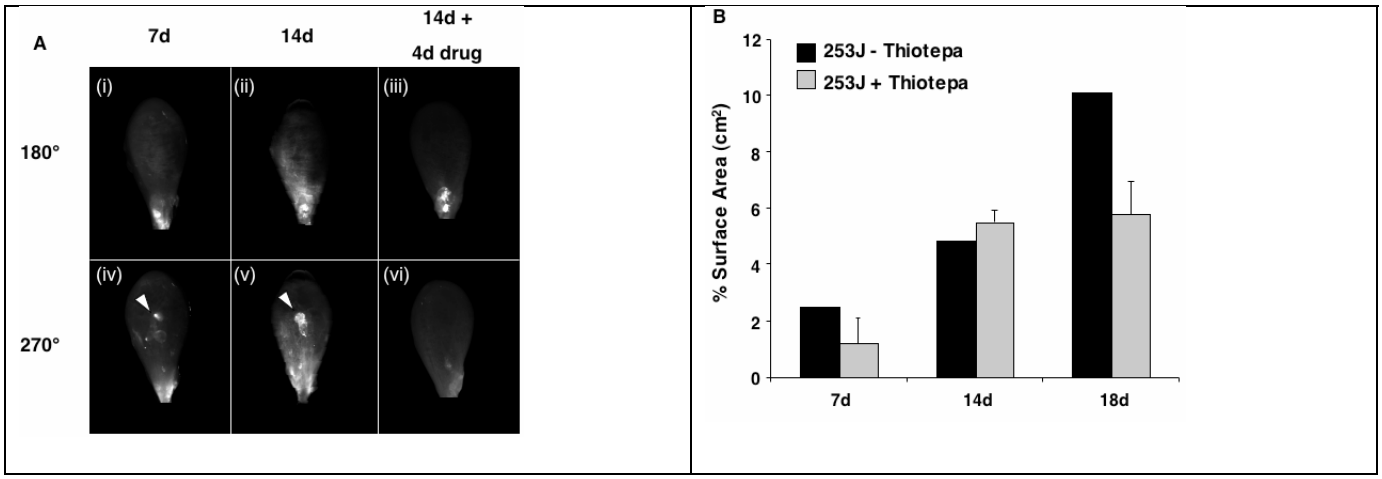

Figure 6: Epifluorescence imaging of Qdot-labeled tumor cells in the whole bladder organ culture model. (A) The figure shows images captured on two aspects (180^o and 270^o) of bladders incubated for 7 d, 14 d or 18 d with 200 µM thiotepa added on d 14 for the last 4 d. (B) Fluorescent areas captured in (A) were measured using Image J and graphed.

2.3.2 System Developments for Continuous Anesthesia and Prolonged Measurements in Mice

Our *in vivo* optical imaging apparatus has been refined to support continuous inhalation anesthesia for an indefinite duration. We have tested this in an application involving the measurement of CSF flow in mice. Animals were anesthetized and then injected with a mixture of indocyanine green (ICG) and human high-density lipoprotein (HDL) into the subarachnoid space at L5. The experiment is illustrated in brief in Figure 7. The mouse was held by a combination bite-block/anesthesia mask, which was fixed to the stage. Closed-loop temperature control was employed to maintain body temperature and breathing rate was monitored by a pressure sensor. The results of an experiment with a mouse under continuous inhalation anesthesia for a six hour duration are shown in the figure. Images were acquired at intervals of 10 minutes and fluorescence signal intensity is shown in the graph of Figure 7C for the region of interest set shown in Figure 7B.

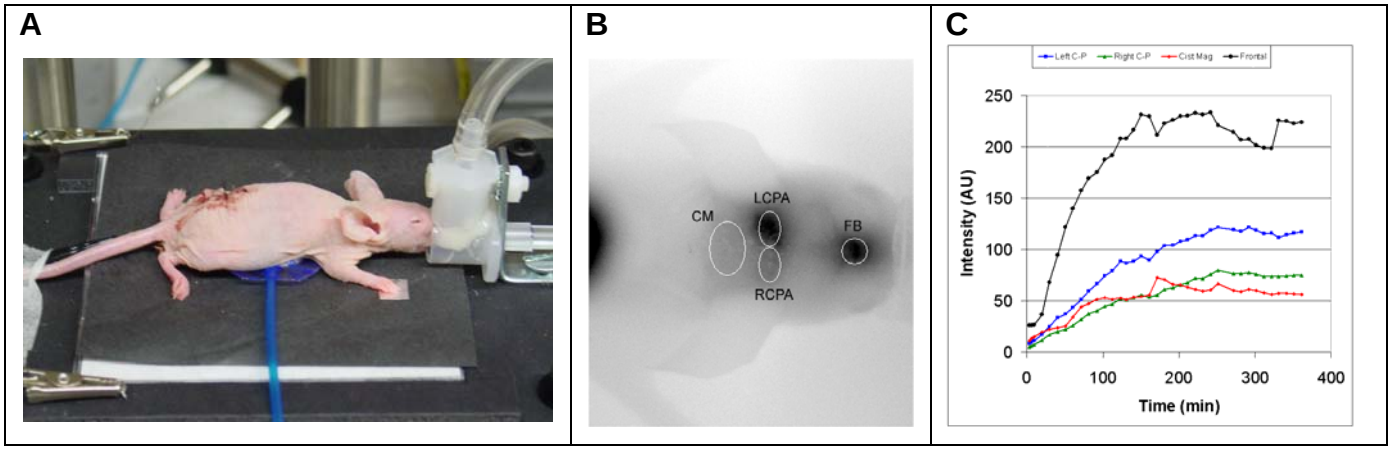

Figure 7: Dynamic cerebral NIR fluorescence imaging. (A) Mouse in position on the system stage for dynamic imaging under continuous inhalation anesthesia. Mouse is under closed-loop temperature control, the respiratory rate is monitored, and a bite bar secures the head. (B) Gray scale fluorescence intensity image at 200 minutes post injection overlaid with ROIs. Regions are chosen to correspond to external projection of the cisterna magna (CM), left and right cerebellopontine angles (LCPA, RCPA), and the frontal base (FB). (C) Time-intensity curves for the four regions of interest as shown in B.

3. Key Research Accomplishments

The overall objective of the proposal, as originally stated in the application, was "**to develop biochemical markers for calcium that serve as contrast agents in advanced imaging procedures.**" We have achieved this objective on a number of fronts. In particular:

- We have developed and refined a fluorophore-based calcium imaging compound based on alendronate, an amino bisphosphonate with a high affinity for calcium. We have tested this compound by injecting it into mice and demonstrated binding to calcium targets.
- We have developed and optimized an optical imaging system that serves as a test-bed for developing and evaluating the agents formulated with optical tags. The system is capable of high resolution, high-sensitivity imaging of small animals *in vivo*. We have employed the system to evaluate fluorescence-tagged experimental contrast agents including the alendronate-fluorophore complex developed here.

- Beyond the stated objectives, driven by productive collaborative effort, we have refined the technology developed here and extended its utility in novel applications for imaging in cancer research.

4. Reportable Outcomes

This research endeavor has so-far resulted in the following reportable outcomes. Other manuscripts are still in preparation.

1. Y Shibata, JB Kruskal, MR Palmer, "Imaging of CSF space and movement in mice using near infrared fluorescence." *J Neursci Meth*, 147(2), 82–87, 2005.
2. Carlos R. Estrada, Matthew Salanga, Diane R. Bielenberg, W. Bruce Harrell, David Zurakowski, Xuping Zhu, Matthew R. Palmer, Michael R. Freeman and Rosalyn M. Adam., "Behavioral profiling of human transitional cell carcinoma *ex vivo*." *Cancer Res*, 66(6), 3078–3086, 2006.

5. Conclusions

In this work, we have developed and optimized an optical imaging system that allows the evaluation of fluorescence-tagged contrast agents to be visualized in living mice. We have synthesized an alendronate-fluorophore conjugate that shows strong affinity for physiological calcium and we have demonstrated the agent in whole-body *in vivo* imaging experiments in mice. This agent or its derivatives may find utility as contrast agents for radiological examinations in the human breast. While developing the system and its application in fluorescence visualization, a number of spin-off technologies were created. The work described here is part of our laboratory's ongoing effort to develop apparatus and techniques for advanced imaging applications with animal models. The support received through the BCRP initiative has been critical in enabling us to test concepts in the early stages of development. Applications consisting of proposals to build on these innovations, initiated by the PI and by our collaborators, have been submitted to other agencies iincluding NIH and ACS.

References

[1] S. H. Heywang-Köbrunner, D. D. Dershaw, and I. Schreer, *Diagnostic Breast Imaging: Mammography, Sonography, Magnetic Resonance Imaging, and Interventional Procedures*, 2nd Ed. ed. New York: Thieme, 2001.

[2] M. J. Rogers, S. Gordon, H. L. Benford, F. P. Coxon, S. P. Luckman, J. Monkkonen, and J. C. Frith, "Cellular and molecular mechanisms of action of bisphosphonates," *Cancer*, vol. 88, pp. 2961-2978, 2000.

[3] O. J. Degrossi, P. Oliveri, H. G. del Río, R. Labriola, D. Artagaveytía, and E. B. Degrossi, "Technitium-99m ADP compared with technetium-99m MDP as a bone scanning agent," *J Nucl Med*, vol. 26, pp. 1135-1139, 1985.

[4] S. Sherman, "Preventing and treating osteoporosis: Strategies at the millennium," *Ann NY Acad Sci*, vol. 949, pp. 188-197, 2001.

[5] R. Maisano, S. Pergolizzi, and S. Cascinu, "Novel therapeutic approaches to cancer patients with bone metastasis," *Crit Rev Oncol Hematol*, vol. 40, pp. 239-250, 2001.

[6] A. Zaheer, R. E. Lenkinski, A. Mahmood, A. G. Jones, L. C. Cantley, and J. V. Frangioni, "*In vivo* near-infrared fluorescence imaging of osteoblastic activity," *Nat Biotech*, vol. 19, pp. 1148-1154, 2001.